Indian Journal of Experimental Biology
Vol. 38, September 2000, pp. 877-880

Antioxidant activity of tannoid principles of *Emblica officinalis* (amla) in chronic stress induced changes in rat brain

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Received 7 July 1999; revised 29 May 2000

Effect of tannoid principles emblicanin A, emblicanin B, punigluconin, and pedunculagin of *E. officinalis* was assessed on chronic unpredictable footshock-induced stress-induced perturbations in oxidative free radical scavanging enzymes in rat brain frontal cortex and striatum. Chronic stress, administered over a period of 21 days, induced significant increase in rat brain frontal cortical and striatal superoxide dismutase (SOD) activity, concomitant with significant reduction in catalase (CAT) and glutathione peroxidase (GPX) activity. The changes in the enzyme activities was accompanied by an increase in lipid peroxidation, in terms of augmented thiobarbituric acid-reactive products. Administration of Emblica tannoids (10 and 20 mg, po) for 21 days, concomitant with the stress procedure, induced a dose-related alteration in the stress effects. Thus, a tendency towards normalization of the activities of SOD, CAT and GPX was noted in both the brain areas, together, with reduction in lipid peroxidation. The results indicate that the reported antistress *rasayana* activity of *E. officinalis* may be, at least partly due to its tendency to normalize stress-induced perturbations in oxidative free radical scavenging activity, in view of the postulate that several stress-induced diseases, including the process of aging, may be related to accumulation of oxidative free radicals in different tissues.

Emblica officinalis Gaertn., commonly known as *amla,* is extensively found all over India, in Sri Lanka, Malaysia, China, Pakistan and Bangladesh The fruits of the plant are used in Ayurveda as a potent *rasayana*[1,2]. The *rasayanas* are used to promote health and life-span by increasing defence against disease, arresting the aging process and revitalizing the body in debilitated conditions[2]. The clinical efficacy of the fruits of *E. officinalis* are held in high esteem in Ayurveda and *amla* is referred to as a maharasayana[2]. The fruits form the major constituent of *Chayavanprash awaleha*, a polyherbal Ayurvedic *rasayana* preparation described in Charaka Samhita[3]. This preparation is widely used in this country for its preventive, curative and health restorative properties. Experimental studies conducted with the fruits indicate that they have significant effect against isoprenaline-induced myocardial injury, radiation-induced chromosomal damage and heavy metal induced liver and renal damage[4]. Clinical studies suggest that the fruits have anabolic activity[4]. Experimental investigations on *Chyavanprash* have indicated that it exhibits significant adaptogenic, anti-stress, immunopotentiating and memory-facilitating effects[4]. It is postulated[5] that several of the stress-induced diseases, including the process of aging, may be related to the accumulation of oxidative free radicals, commonly referred to as reactive oxygen species (ROS), in different tissues. *Emblica officinalis* tannoids have recently been reported to enhance ROS scavenging activity in rat brain frontal cortex and striatum, enhancing the concentrations of the antioxidant enzymes superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX), resulting in reduced lipid peroxidation[6]. In the present study, the effects of reported active tannoids of *E. officinalis*[7] have been investigated on chronic stress-induced perturbations in SOD, CAT GPX and lipid peroxidase (LPO) activities in rat brain frontal cortex and striatum. These brain areas were selected in view of their reported vulnerability to oxidative stress-induced degeneration[5].

Materials and Methods

The study was conducted on adult male CF strain rats (160-180 g). The animals were housed in colony cages at an ambient temperature of $25^0 \pm 2^0$C and 45-55% RH, with 12; 12 hr L; D cycle. They had free access to standard pellet chow and drinking water. Experiments were conducted between 0900 and 1400 hrs.

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The test compound, was prepared from fresh juice of *E. officinalis* fruits by deactivating the contained hydrolytic enzymes followed by column chromatography over Sephadex LH-20, methanol and methanol-water as eluent. The concentrations of emblicanin A (37%), emblicanin B (33%), punigluconin (12%), pedunculagin (14%), rutin (3%) and gallic acid (1%) in the extract were established by HPTLC, using authentic markers[7]. Details of extraction and structure elucidation of the emblicanins and other compounds from *E. officinalis* fruit juice have been published earlier[7].

The method of Conti *et al*[8], with some modifications required to add an element of unpredictability to the footshock-induced stress, was used. The rats were subjected to daily 1 hr footshock through a grid floor in a perspex box for 21 days. The duration of each shock (2 mA) and the intervals between the shocks was randomly programmed between 3-5 sec and 10-110 sec, respectively. The standardized *E. officinalis* extract (EOT) was dissolved in 0.9% saline and administered po, in the doses of 10 and 20 mg/kg, once daily for 21 days, 1 hr prior to the footshock stress. Control animals received an equivalent value of 0.9% saline (1ml/kg) through the same route and for the same time period. The rats were sacrificed by decapitation on day 21, 1 hr after the last footshock exposure in both the drug- and saline-treated groups. The brains were removed and the frontal cortex and striatum dissected out[9]. The brain tissues were weighed, homogenized in 2 ml of ice-cold triple distilled water and sonicated for 16 sec. The homogenates were then centrifuged (10,000 *g*, 2 min) and the supernatants were used for the biochemical estimations. However, for the estimation of lipid peroxidation, the tissues were homogenized in cold potassium chloride (1.15%) solution. The following methods were used :

SOD activity—The assay was based on the ability of SOD to inhibit the spontaneous oxidation of adrenaline to adenochrome[10]. Results are expressed as units (U) of SOD activity/mg protein. One unit of SOD activity induced approximately 50% inhibition of auto-oxidation of adrenaline.

CAT activity—The assay was based on the ability of CAT to induce the disappearance of hydrogen peroxide (H_2O_2), which was followed spectrophotometrically[11]. One unit (U) of CAT was defined as the amount of the enzyme required to decompose 1 μmol of H_2O_2 per min, at 25^0 C and pH 7.0 Results are expressed as units (U) of CAT activity/mg protein.

Glutathione peroxidase activity—H_2O_2 was used as the substrate. Sodium azide (1m*M*) was added to the reaction mixture in order to inhibit remnant CAT activity[12]. One unit of GPX was defined as the amount of the enzyme decomposing 1μmol H_2O_2 /min, at 25^o and *pH* 7. Results are expressed as units (U) of GPX activity/mg protein.

Lipid peroxidation (LPO)—LPO was determined by estimating the accumulation of the peroxidative product, thiobarbituric acid-reactive substances (TBARS), using a standard curve of 1,1,3,3-tetramethoxypropane, and was expressed as nmol TBRAS/gm brain tissue[13].

Protein estimation—was done by the method of Lowry *et al*,[14]

The data were analysed by the Mann-Whitney U-test. $P < 0.05$ was considered statistically significant.

Results and Discussion

The results are summarized in Table 1.

EOT (10 and 20 mg/kg, po x 21 days) significantly increased frontal cortical and striatal SOD, CAT and GPX concentrations with concomitant reduction in LPO values. Footshock stress, administered over a period of 21 days, induced marked increase in frontal cortical (107.6%) and striatal (87.0%) SOD concentrations with concomitant decreases in frontal cortical CAT (53.0%) and GPX (50%), and striatal CAT (57.3%) and GPX (50%) activities. LPO values, expressed as accumulation of TBRAS, were markedly increased both in frontal cortex (91.2%) and striatum (82.7%). Treatment of the rats with EOT for 21 days in the stressed animals, in the doses of 10 and 20 mg/kg, po, tended to reverse, in a dose-dependent manner, the stress induced changes in SOD, CAT, GPX and LPO activities in the rat brain. Thus, EOT was found to reduce the stress-induced augmented SOD and LPO activities, and to reverse stress-induced decrease in CAT and GPX activities in both the brain areas investigated.

E. officinalis has been subjected to extensive chemical investigations. It was earlier believed that the biological and therapeutic effects of *amla* fruits was due to their rich vitamin C (L-ascorbic acid) content, said to range from 0.1 to 0.7% in fresh pericarp[7]. However, more recent studies[7] have indicated the complete absence of L-ascorbic acid in the fresh juice and solvent extractives of the plant fruits. The investigations further indicated that the potent vitamin C-like activity of the fruits was due to the presence of low molecular weight hydrolysable tannoids. Four such compounds, emblicanin A, emblicanin B, punigluconin and pedunculagin, were isolated from fresh pericarp and their chemical structures were

Table 1—Effects of *Emblica officinalis* tannoid principles (EOT) on chronic stress (CS) induced perturbations in rat brain superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX) and lipid peroxidase (LPO) activity

[Values are mean±SEM]

Treatment (mg/kg, p.o.)	n	SOD (U/mg protein)	CAT (U/mg protein)	GPX (U/mg protein)	LPO (nmol TBARS/gm)
Frontal cortex					
Vehicle	12	18.4±2.2	20.4±2.0	0.08±0.004	2.96±0.6
EOT (10)	8	24.2 ± 1.6^{x}	24.6 ± 0.8^{x}	0.12 ± 0.009^{x}	1.72 ± 0.4^{x}
EOT (20)	8	26.8 ± 1.2^{y}	29.2 ± 1.0^{y}	0.18 ± 0.06^{y}	1.34 ± 0.6^{z}
Vehicle + CS	12	38.2 ± 2.9^{z}	9.6 ± 0.8^{z}	0.04 ± 0.003^{z}	5.66 ± 0.9^{z}
EOT (10) + CS	8	23.3 ± 2.8^{b}	12.6 ± 0.9^{a}	0.06 ± 0.004^{a}	3.94 ± 0.8^{a}
EOT (20) + CS	8	29.4 ± 2.6^{b}	15.9 ± 0.6^{c}	0.08 ± 0.006^{c}	2.83 ± 0.9^{b}
Striatum					
Vehicle	12	22.4±0.9	24.8±1.2	0.12±0.006	3.24±0.8
EOT (10)	8	26.9 ± 1.4^{x}	29.2 ± 0.6^{x}	0.18 ± 0.007^{x}	2.16 ± 0.6^{x}
EOT (20)	8	31.4 ± 1.1^{y}	32.0 ± 1.2^{y}	0.22 ± 0.012^{y}	1.84 ± 0.8^{y}
Vehicle + CS	12	41.9 ± 1.6^{z}	10.6 ± 0.6^{z}	0.06 ± 0.004^{z}	5.92 ± 0.7^{z}
EOT (10) + CS	8	25.4 ± 0.8^{c}	14.6 ± 0.6^{a}	0.09 ± 0.004^{a}	3.96 ± 0.9^{a}
EOT (20) + CS	8	20.4 ± 0.8^{c}	20.6 ± 0.8^{c}	0.11 ± 0.005^{c}	3.28 ± 0.8^{b}

EOT was administered once daily concomitantly with once daily foot-shock stress

P values : $^{x} < 0.05$; $^{y} < 0.01$; $^{z} < 0.001$ different from vehicle-treated group; $^{a} < 0.05$; $^{b} < 0.01$; $^{c} < 0.001$ different from chronic stress (CS) group (Mann-Whitney U-test).

established by spectroscopic analysis and chemical transformation[7]. Emblicanin A and B were also shown to exhibit significant antioxidant activity *in vitro*[7]. The *in vivo* antioxidant effect of the *E. officinalis* fruit tannoids was confirmed in a later study[6], in which they were shown to augment SOD, CAT and GPX activities in frontal cortex and striatum of rats, concomitant with reduction in LPO activity in these brain areas.

It is now widely accepted that mild chronic stress is clinically more relevant than the hitherto commonly used acute stress models[15]. Making the mild chronic stress unpredictable adds to its experimental utility, as was noted by the induction of behavioural states used as animal models of clinical depression[15]. Recent studies, using mild unpredictable chronic footshock, indicate that this form of stress can induce several physiological, biochemical and behavioural perturbations in rats, including glucose intolerance, increase in plasma corticosterone, peptic ulceration, immunosuppression, cognitive deficits, behavioural depression and male sexual dysfunction[16,17]. It is postulated that several of the stress-induced diseases may be due to oxidative stress brought about by either increased generation of ROS or decreased free radical scavenging activity[18].

It is of interest to note that although EOT has been shown to augment SOD activity in frontal cortex and striatum of unstressed rats[6], it tended to normalize stress induced increase in SOD activity in these regions. Cold-restraint stress gastric ulcers in rats have been correlated with increased gastric mucosal SOD and LPO activity[19]. Administration of antioxidants like reduced glutathione or sodium benzoate prior to stress, caused significant decrease in gastric ulceration concomitant with reduced LPO activity[19]. An increase in SOD and LPO activity has been demonstrated in the cortex, cerebellum and hippocampus of aged rats[20], consonant with the oxidative free radical hypothesis of aging[20]. Long term administration of a biocatalyzer, said to have antiaging effect, reduced LPO which was accompanied by a decrease in the augmented SOD activity[20].

The superoxide anion ($O_2^{\cdot-}$), H_2O_2 and hydroxyl radical ($OH^{\cdot}$) are the major ROS which induce cell degeneration by increasing LPO of cell membrane lipids. The toxic end products of peroxidation induce damage of the structural and functional integrity of cell membranes, break DNA strands and denature cellular proteins[21]. The natural cellular antioxidant enzymes include SOD, which scavenges superoxide radicals by speeding up their dismutation, CAT, a haeme enzyme which removes H_2O_2, and GPX, a selenium-containing enzyme which scavenges H_2O_2 and other peroxides[21]. Detoxification of the superoxide anion is not a terminating step in free radical scav-

enging, since the enzyme-catalysed dismutation results in the production of H_2O_2 which accumulates in the mitochondria and cytosol. Unless the peroxide is scavenged by CAT and GPX, it, in the presence of iron, may also lead to production of $OH^{\bullet}$. These ROS, together with singlet molecular oxygen, may attack lipids, proteins and DNA of cells following increased lipid peroxidation chain reactions resulting in widespread cellular injury[21]. It is possible that, in chronic stress, the increased levels of SOD leads to increased generation of peroxides which, due to reduced CAT and GPX levels, are not effectively scavenged resulting in augmented lipid peroxidation. EOT appears to mitigate these chronic stress-induced effects, tending to normalize SOD activity and increasing the activities of CAT and GPX. These stress effects will, therefore, lead to decreased LPO and attenuation of the adverse effects of chronic stress.

The results of the present investigation support the postulate that increased ROS induced by chronic stress may be involved in at least some of the aversive effects of stress. *Rasayanas*, like *E. officinalis*, may be clinically effective in stress induced pathological states due to their effects on oxidative free radical scavenging enzymes and resultant attenuated lipid peroxidation. The results also confirm that the low molecular tannoid principles of *E.officinalis* are responsible for the anti-oxidative activity of the fruits of the plant. The emblicanins are likely to be the major antioxidant principles, not only because they are the major constituents of *E. officianalis* but also because of their reported antioxidant actions *in vitro*[7] and *in vivo*[6,22].

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